78. A method for treating a patient having a disease or medical condition characterized by abnormalities of nitric oxide and oxygen metabolism, comprising administering to the patient an effective amount of a composition comprising nitrosylhemoglobin.

79. A method for producing a composition comprising SNO-methemoglobin, comprising incubating excess S-nitrosothiol with purified methemoglobin at pH 7.4 to 9.2.

REMARKS

Claims 10, 13, 24-26 and 66 have been canceled. Claims 11, 14, 46, 63 and 65 have been amended. Claims 67-77 have been added.

Claims 67-72 are derived from original Claims 10-15, respectively. Support for Claims 67-69 can be found on page 47, line 14 to page 48, line 13, on page 48, line 34 to page 49, line 8, and on page 69, lines 21-28. Support for Claims 70-72 can be found on page 47, lines 1-5, for example.

Claims 73-76 are derived from Claims 20-22 and 27, respectively. Support for Claims 73-76 can be found in Example 15, page 72, line 21 to page 73, line 8, in Example 18, page 75, lines 5-28, page 28, line 34 to page 29, line 15, and on page 30, lines 24-33, for instance.

Rejection of Claims 63, 65 and 66 Under 35 U.S.C. § 112, First Paragraph (Item 2 of Office Action)

Claims 63, 65 and 66 have been rejected under 35 U.S.C. § 112, first paragraph, as it is said that they contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the claimed invention. Claim 66 has been canceled.

The Examiner states, "The specification disclosure (bottom of page 72 - top of 73) of a specific multistep protocol for forming SNO-hemoglobin does not support the generic method claim 63." As amended, Claim 63 is drawn to a method having two steps, with the essential conditions of the steps specified. Methods for producing deoxyhemoglobin are known in the prior art. The method of Claim 63 is described on page 72, lines 23-31, and in Figure 13, wherein the yield of SNO-hemoglobin is shown to be significant at a heme:NO ratio of less than 10.

The Examiner states, "The specific disclosure (bottom of page 75 - top of page 76) which discloses a specific multistep protocol for making SNO-Hb which includes specific buffer and amounts does not support the generic method of new claim 65. Additionally, there is no support for the newly added language (e.g., 5/1/00 amendment) e.g. 'At a heme:NO ratio of less than about 10.'"

The method described on page 75, line 30 to page 76, line 1, that the Examiner refers to as a specific multistep protocol, starts with purified oxyhemoglobin, which can be prepared in a number of ways known in the prior art. The method is only one step -- to add NO dissolved in an aqueous solution (wherein the NO solution has been stored under nitrogen to prevent oxidation of the NO, as one of skill in the art would know) to the oxyhemoglobin in the presence of oxygen, at pH 7.4. Figure 17 shows that the yield of SNO-hemoglobin is significant at a heme:NO ratio of less than 10.

Every detail of the methods does not need to be provided in the claims. The written description and figures give examples of exact conditions that were successful, and other variations of these conditions could be easily derived from the examples. No person skilled in the art would assume, for example, that the success of the methods depends on only one type of buffer or one incubation time. In this synthesis, NO binds initially to Fe to form nitrosylhemoglobin, which is known to occur under a wide variety of conditions; then, nitrosylhemoglobin is converted to SNO-hemoglobin. An assay for SNO-hemoglobin is provided in the written description at page 66, line 11 to page 68, line 4 so that the success of the SNO-hemoglobin synthesis method can be confirmed.

Rejection of Claims 10-15, 46, 63, 65 and 66 Under 35 U.S.C. § 112, First Paragraph (Item 3 of Office Action)

Claims 10-15, 46, 63, 65 and 66 have been rejected under 35 U.S.C. § 112, first paragraph, as "based on a disclosure which is not enabling."

Claims 10, 13 and 66 have been canceled. Claims 11, 14, 46, 63 and 65 have been amended to more clearly define the reaction conditions. The pH of the reaction conditions has been added to the claims according to the specification on page 49, lines 4-8. Examples of conditions for producing SNO-hemoglobin (both oxy- and deoxy-) are given on page 49, lines 19-27 of the written description. See also page 57, lines 18-22 for the preparation of SNO-

oxyhemoglobin. For a further example of the preparation of a composition comprising SNO-oxyhemoglobin, see page 69, lines 22-24. For the preparation of SNO-methemoglobin, see page 57, lines 18-26. It can be seen from these portions of the written description that the essential method parameters are present in the disclosure, providing one of ordinary skill in the art with sufficient guidance to carry out the methods of the claims. An assay for SNO-hemoglobin is provided in the written description at page 66, line 11 to page 68, line 4 so that the success of the SNO-hemoglobin synthesis method can be confirmed.

Rejection of Claims 10-14, 43, 44, 63 and 66 Under 35 U.S.C. § 102(b), Or, Alternatively, Under 35 U.S.C. § 103 (Item 4 of Office Action)

Claims 10-14, 43, 44, 63 and 66 have been rejected under 35 U.S.C. § 102(b) as being anticipated by Wade and Castro, or, alternatively, under 35 U.S.C. § 103 as being obvious over Wade and Castro. Claims 10, 13 and 66 have been canceled.

Wade and Castro (*Chem. Res. Tox. 3*:289-291, 1990) describe reactions of metmyoglobin and other oxidized heme proteins, including methemoglobin, with NO and certain organic nucleophiles, yielding nitroso products of the organic nucleophiles, and not of hemoglobin. SNO-hemoglobin is not reported as a product, nor is any other SNO- derivative of a heme protein reported as a product. The synthesis of SNO-hemoglobin is not anticipated, not looked for, and not found by Wade and Castro.

Claims 11 and 12 are not the method of the Wade and Castro reference because the method of Claims 11 and 12 is carried out in the presence of oxygen, while the reaction of Wade and Castro is carried out in anaerobic conditions. The step in the method of Claims 11 and 12 requires incubating excess nitrosating agent with purified hemoglobin in the presence of oxygen. The step of the method of Wade and Castro requires incubating excess NO with purified heme protein, such as hemoglobin, *with a nucleophile such as phenol*, in the absence of oxygen. The addition of the nucleophile makes this incubation step different from that of the method of Claims 11 and 12. Thus, the method of Claim 11 is different in four ways from the method of Wade and Castro: 1) Claim 11 is a method for nitrosating hemoglobin, not an added nucleophile; 2) Claim 11 uses reduced proteins; Wade and Castro use oxidized proteins; 3) Claim 11 uses aerobic conditions; Wade and Castro use anaerobic conditions; 4) Claim 11 has no added nucleophile; Wade and Castro add an organic nucleophile which is nitrosated. In

addition, in Claim 12 the reagent is a low molecular weight S-nitrosothiol, not NO, as is used in Wade and Castro.

Claim 14 is not the method of the Wade and Castro reference because the method of Wade and Castro requires incubating excess NO with a purified oxidized heme protein, such as methemoglobin, *with a nucleophile such as phenol*, in the absence of oxygen. The addition of the nucleophile makes this incubation step different from the step in the method of Claim 14. Thus, the method of Claim 14 is different in three ways from the method of Wade and Castro: 1) Claim 14 is a method for nitrosating hemoglobin, not an added nucleophile; 2) Claim 14 uses reduced proteins; Wade and Castro use oxidized proteins; 3) Claim 14 has no added nucleophile; Wade and Castro add an organic nucleophile which is nitrosated.

Claims 43 and 44 are not the method of the Wade and Castro reference because the method of Claim 43 is carried out in the presence of oxygen with reduced hemoglobin, while the reaction of Wade and Castro is carried out in anaerobic conditions with oxidized hemoglobin. The step in the method of Claim 43 and 44 requires adding NO to an aqueous solution of oxyhemoglobin (which will only be in oxyhemoglobin form in the presence of oxygen). The step of the method of Wade and Castro requires incubating excess NO with purified oxidized heme protein, such as methemoglobin, *with a nucleophile such as phenol*, in the absence of oxygen. The addition of the nucleophile makes this incubation step different from that of the methods of Claims 43 and 44. Thus, the methods of Claims 43 and 44 are different in four ways from the method of Wade and Castro: 1) Claims 43 and 44 are methods for nitrosating hemoglobin, not an added nucleophile; 2) Claims 43 and 44 use reduced proteins; Wade and Castro use oxidized proteins; 3) Claims 43 and 44 use aerobic conditions; Wade and Castro use anaerobic conditions; 4) Claims 43 and 44 have no added nucleophile; Wade and Castro add an organic nucleophile which is nitrosated.

Claim 63 is not the method of the Wade and Castro reference because the step of the method of Wade and Castro requires incubating excess NO with purified oxidized heme protein, such as methemoglobin, *with a nucleophile such as phenol*, in the absence of oxygen. The addition of the nucleophile makes this incubation step different from that of the mixing step of Claim 63. Claim 63 has a second step of "exposing the resulting solution to air," which is not described in Wade and Castro. Thus, the method of Claim 63 is different in four ways from the method of Wade and Castro: 1) Claim 63 is a method for nitrosating hemoglobin, not an added

nucleophile; 2) Claim 63 uses reduced proteins; Wade and Castro use oxidized proteins; 3) Claim 63 has a second step of exposing the resulting solution to air; Wade and Castro do not describe such a step; 4) Claim 63 has no added nucleophile; Wade and Castro add an organic nucleophile which is nitrosated.

Claim 65 is not the method of the Wade and Castro reference because the method of Claim 65 is carried out in the presence of oxygen, while the reaction of Wade and Castro is carried out in anaerobic conditions. The step in the method of Claim 65 requires mixing nitric oxide and oxyhemoglobin (which will only be in oxyhemoglobin form in the presence of oxygen). The step of the method of Wade and Castro requires incubating excess NO with purified heme protein, such as hemoglobin, *with a nucleophile such as phenol*, in the absence of oxygen. The addition of the nucleophile makes this step different from the step in the method of Claim 65. Thus, the method of Claim 65 is different in four ways from the method of Wade and Castro: 1) Claim 65 is a method for nitrosating hemoglobin, not an added nucleophile; 2) Claim 65 uses reduced proteins; Wade and Castro use oxidized proteins; 3) Claim 65 uses aerobic conditions; Wade and Castro use anaerobic conditions; 4) Claim 65 has no added nucleophile; Wade and Castro add an organic nucleophile which is nitrosated.

The Examiner states that the Wade and Castro reference "discloses a method of transferring the nitrosyl group to sulfur (as well as oxygen, nitrogen and sulfur) of heme proteins, including hemoglobin to thus form SNO-hemoglobin . . . ." The Wade and Castro reference does not describe products resulting from reaction at the oxygen, nitrogen or sulfur atoms of hemoglobin or any other heme protein. The only product described in the reference as resulting from a reaction of NO with heme proteins is the heme protein with a "heme-NO" adduct, that is, with NO bonded to the Fe of the heme (see equation (2) on page 289 of Wade and Castro). In the case of hemoglobin, the product postulated by Wade and Castro is nitrosylhemoglobin (in which NO is bound to the heme Fe), not SNO-hemoglobin. The other products described in the reference are those resulting from NO reacting with the added reagent having a nucleophilic site – N-acetylcysteine, phenol, or proline.

The Examiner states that ". . . it would have been obvious to one of ordinary skill in the art to generate the oxygenated hemoglobin species by air oxidation especially since the reference specifically points to a nitrosation process which occurs under aerobic conditions." The Examiner appears to be referring to a reference cited in Wade and Castro which describes

nitrosation (in the presence of air) of diphenylamine by nitrosylhemoglobin. [See page 290, first column, lines 17-22, below equation (3)]. SNO-hemoglobin is not a product of the process referred to here, nor is SNO-hemoglobin (oxy- or deoxy-) a product of any other process described in or referred to in the Wade and Castro reference. SNO-hemoglobin and other SNO heme proteins are not mentioned at all for any reason.

The Examiner states that ". . . the reference is practicing the SAME METHOD STEPS as presently claimed." This is incorrect. The method of the reference differs from the methods of Claims 11, 12, 14, 43, 44, 63 and 65 for the reasons stated above. All of the reactions described in or referred to in the Wade and Castro reference include an organic compound which has a nucleophilic site where NO reacts to produce a C-NO, N-NO or S-NO adduct, depending on the organic compound included in the reaction. See Scheme I on page 289 of Wade and Castro. None of the methods of Claims 11, 12, 14, 15, 43, 44, 63 or 65 include a step in which the reactants can include more than hemoglobin and nitrosating agent. Thus, the reactions described in Wade and Castro do not anticipate the claimed methods.

The Wade and Castro reference also does not suggest the methods of Claims 11, 12, 14, 15, 43, 44, 63 or 65. There is no suggestion to alter the reaction conditions by leaving out the organic nucleophile, to produce any alternative products. Nowhere is it suggested that NO can be an adduct on a heme protein anywhere but at the heme Fe. The object of Wade and Castro was to produce SNO- low molecular weight organic nucleophiles, not SNO-hemoglobin. To do this, Wade and Castro avoid the conditions used in the methods of the Claims.

Rejection of Claims 16, 20-22, 27, 28 and 40 Under 35 U.S.C. § 103(a) (Item 5 of Office Action)

Claims 16, 20-22, 27, 28 and 40 have been rejected under 35 U.S.C. § 103(a), as they are said to be obvious over Stamler *et al.*, WO 93/09806.

Stamler *et al.* (WO 93/09806) disclose S-nitroso-proteins, in particular, S-nitroso-tPA (tPA is tissue plasminogen activator), S-nitroso-BSA, S-nitroso-cathepsin B, S-nitroso-lipoprotein and S-nitroso-immunoglobulin, and methods for producing the same, using NO or $NaNO_2$ as the reagent under acidic conditions. They also report a method which they claim results in the synthesis of S-nitroso-hemoglobin. However, this compound was not produced by any method reported in WO 93/09806, as attested to in the Declaration of Jonathan S. Stamler,

M.D. Under 37 C.F.R. § 1.132 mailed to the Patent Office on January 6, 1999. Methods used to synthesize other S-nitroso-proteins, which might have been expected to nitrosate or polynitrosate hemoglobin, dissociated hemoglobin into its subunits, oxidized the heme Fe and rendered the product fragments useless for carrying oxygen. Methods described in the specification that resulted in the synthesis of nitrosated hemoglobins are substantially different from the unsuccessful acidified nitrite method described in WO 93/09806.

Claim 16

Unmodified purified hemoglobin has been administered alone as a carrier of oxygen, and is known to be a vasoconstrictor. See, for example, Feola *et al.*, (US 5,439,882) last sentence of column 4.

One species of nitrosated hemoglobin that had been characterized at the time of the invention was nitrosylhemoglobin, which has an NO adduct on the heme Fe. Nitrosylhemoglobin, having NO bound at the heme Fe instead of having O_2 bound, is useless for carrying oxygen and is nowhere reported in the prior art as being a donor of NO. Thus, one of ordinary skill in the art would think it useless for any "method for regulating delivery of oxygen and NO in a mammal" as called for in Claim 16. One of ordinary skill in the art would have no reason to use nitrosylhemoglobin alone or in any combination in the method of Claim 16, where the objective is to regulate delivery of oxygen and NO in a mammal.

Applicants have shown that nitrosylhemoglobin can be converted under physiological conditions in the lung to SNO-hemoglobin. See Example 15, page 72, line 21 to page 73, line 8, Example 18, page 75, lines 5-28, and page 28, line 34 to page 29, line 15. This conversion process was previously unknown.

As has been explained at length previously, WO 93/09806 does not provide an enabling description of how one of ordinary skill in the art could produce SNO-hemoglobin or hemoglobin that has an NO adduct at a C, N, or O. See also the Declaration of Jonathan S. Stamler, M.D. Under 37 C.F.R. § 1.132 mailed to the United States Patent and Trademark Office on January 6, 1999.

One combination in the method of Claim 16 is hemoglobin plus a low molecular weight thiol. This combination is not discussed or implied for any purpose in WO 93/09806. A low

molecular weight thiol is brought up in a description of a method to thiolate a protein (see page 22, lines 24-29 of WO 93/09806), but in no other context.

A second combination in the method of Claim 16 is hemoglobin plus a low molecular weight nitrosothiol. This combination is not discussed or implied for any purpose in WO 93/09806. One low molecular weight nitrosothiol (SNOAc, or S-nitrosoacetylcysteine) is described as a reagent to react with hemoglobin (page 58, lines 17-19 of WO 93/09806), but nowhere is there any suggestion that hemoglobin should be administered to a mammal along with a low molecular weight nitrosothiol for any reason. Before the subject application, hemoglobin was thought to bind with high affinity to any NO released by a nitric oxide donor, with the products methemoglobin and nitrosylhemoglobin resulting (respectively, from oxyhemoglobin and deoxyhemoglobin). See Doyle and Hoekstra, *Journal of Inorganic Chemistry 14*:351-358 (1981; enclosed as Appendix 1), especially page 353.

A third combination in the method of Claim 16 is nitrosated hemoglobin and low molecular weight thiol. This combination is not discussed or implied for any purpose in WO 93/09806. As discussed above, a low molecular weight thiol is brought up in a description of a method to thiolate a protein (see page 22, lines 24-29 of WO 93/09806), but in no other context. No role is discussed for any possible enhancement of the effects of a nitrosated hemoglobin by a low molecular weight thiol.

A fourth combination in the method of Claim 16 is nitrosated hemoglobin and low molecular weight nitrosothiol. This combination is not discussed or implied for any purpose in WO 93/09806. As discussed above, one low molecular weight nitrosothiol (SNOAc, or S-nitrosoacetylcysteine) is described as a reagent to be incubated with hemoglobin (page 58, lines 17-19 of WO 93/09806), but nowhere is there any suggestion that a nitrosated hemoglobin – with an NO adduct at any site -- should be administered to a mammal along with a low molecular weight nitrosothiol for any reason. No role is discussed for any possible additive effect of a nitrosated hemoglobin with a low molecular weight nitrosothiol.

The Examiner states:

> Further it is known in the art that hemoglobin is involved in regulating oxygen metabolism by its ability to bind reversibly to blood oxygen and thus facilitate the capability of blood to transport oxygen to bodily tissues (e.g. see bottom of page 19 - top of page 20). Accordingly, it would have been obvious to combine a low molecular weight thiol or nitrosothiol with either hemoglobin or nitrosated

> hemoglobin to deliver oxygen or NO (e.g. claim 16) since the Stamler reference teaches the use of the same compounds separately to effectuate the same function.

Nowhere does WO 93/09806 teach or suggest that low molecular weight thiols or nitrosothiols are involved in regulating oxygen metabolism, either by themselves or in combination with anything else. Nowhere does WO 93/09806 teach or suggest any combination of a low molecular weight thiol or nitrosothiol with hemoglobin or a nitrosated hemoglobin for any enhanced effect of NO delivery. No additive effect is taught and no intermolecular interaction is taught that could suggest any additive effect.

Claims 20-22, 27 and 28

Claims 20-22, 27 and 28 are all drawn to methods of treating a mammal (a human patient in Claims 27 and 28), comprising administering to the mammal nitrosated hemoglobin (or nitrated hemoglobin, in the case of Claim 21). Nitrosated hemoglobins described in the written description of the application are SNO-hemoglobin, polynitrosated hemoglobin, which can have other sites nitrosated in addition to the S atoms of cysteine residues (e.g., C, N, O), and nitrosylhemoglobin, in which the NO adduct is on the Fe of the heme.

The Examiner states:

> Additionally, the use of nitrosated/nitrated proteins, including nitrosated/nitrated hemoglobin to deliver NO to tissues (e.g. claim 40) in order to effectuate the treatment of abnormalities or diseases which are mediated by nitric oxide and oxygen metabolism (e.g. lung disease, sickle cell anemia, heart disease, high blood pressure etc.) would have been obvious since the reference discloses the use of nitrosated proteins, including nitrosated hemoglobin, to treat such disease states.

The WO 93/09806 reference discloses the making of some S-nitroso derivatives of proteins that can be produced by reaction with acidified nitrite, and specific effects: platelet aggregation, relaxation of smooth airway muscle, and vasodilation. As explained in previous Amendments, S-nitrosohemoglobin cannot be made using acidified nitrite, and insufficient support for any other method of synthesis of S-nitrosohemoglobin is presented in WO 93/09806. Any effects of SNO-hemoglobin reported in WO 93/09806 are mere speculation.

The WO 93/09806 reference does not disclose the use of nitrosylhemoglobin in any method of therapy. Claims 73-76 have been added, drawn to methods of treating medical disorders, comprising administering to a mammal an effective amount of nitrosylhemoglobin.

Claim 40

Claim 40 is drawn to a method for delivering NO or its biological equivalent to tissues in an animal or human, comprising administering to the animal or human an effective amount of one or more nitrosyl-heme-containing donors of NO.

The Examiner does not state what teachings of the reference render this method obvious. Nitrosyl-hemoglobin, which is one nitrosyl-heme-containing donor of NO, is mentioned on page 58, lines 19-21 of WO 93/09806, but is not mentioned as being useful in any method of therapy, or as being under any circumstances a carrier or donor of NO. It was not until the experiments described in Examples 15-18 (pages 72-75 of the written description) that it was known that nitrosylhemoglobin can be converted under physiological conditions in the lung to SNO-hemoglobin, which can be a donor of NO.

Rejection of Claim 41 Under 35 U.S.C. § 103(a) (Item 6 of Office Action)

Claim 41 has been rejected under 35 U.S.C. § 103(a), as it is said to be unpatentable over Stamler WO 93/09806 as applied to Claims 16, 20-22, 27, 28 and 40 above, and further in view of Moore *et al.* (*J. Biol. Chem. 251(9)*:2788-2794, 1976) or Sharma *et al.* (*J. Biol. Chem. 253(18)*:6467-6472, 1978).

The Examiner states,

> The Stamler reference although disclosing the use of nitrosyl-heme containing NO donors to deliver NO or its biological equivalent to tissues (e.g. present claim 40) fails to specifically disclose the use of nitrosylhemoglobin (e.g. dependent claim 41). However, nitrosylhemoglobin compositions are conventionally known in the art. E.g., see the Moore and Sharma references. One of ordinary skill in the art would be motivated to select nitrosylhemoglobin to deliver NO to tissues in view of the Stamler reference which suggests that this compound would be expected to function as an NO-donating compound.

The Stamler reference discusses SNO- derivatives of certain proteins, produced by reaction of the proteins with acidified nitrite [e.g., cathepsin, bovine serum albumin (BSA), tissue plasminogen activator (tPA)]. The reference postulates the production of SNO-hemoglobin. None of these are nitrosyl-heme containing donors of NO. A nitrosyl-heme has NO bound to the Fe in the heme. The reference briefly mentions nitrosyl-hemoglobin on page 58, lines 19-21, but nowhere does it discuss nitrosyl-hemoglobin or any nitrosyl-heme-containing compound as being a donor of NO or its biological equivalent.

Nitrosylhemoglobin is known in the art. However, nowhere in the prior art is nitrosylhemoglobin described as being a donor of NO, or as being useful in any method of therapy. One of ordinary skill in the art, presented with the teachings of WO 93/09806 and Moore et al. or Sharma *et al.*, would know how to produce nitrosylhemoglobin in the laboratory, but would know from the teachings of Sharma *et al.* and from other prior art that NO is very tightly bound to the heme Fe, and that nitrosylhemoglobin could not be useful as an NO donor. It was only with the experiments described in Examples 15 and 18 of the subject written description, for example, that it was discovered that nitrosylhemoglobin was converted in the lung to SNO-hemoglobin which is, in fact, a donor of NO.

Rejection of Claims 10-15 Under 35 U.S.C. § 103(a) (Item 7 of Office Action)

Claims 10-15 have been rejected under 35 U.S.C. § 103(a), as they are said to be unpatentable over Stamler *et al.* (WO 93/09806).

Claims 10 and 13 have been canceled.

WO 93/09806 describes a method for producing SNO-hemoglobin. However, this method is described so incompletely that one of ordinary skill in the art could not follow the protocol. No method of assay is described that could measure whether any SNO-hemoglobin had been formed, in fact. The method is merely hypothetical; no evidence is given that SNO-hemoglobin was actually produced, and no physiological effect of SNO-hemoglobin was measured. See the Declaration of Jonathan S. Stamler, M.D. Under 37 C.F.R. § 1.132 mailed to the United States Patent and Trademark Office on January 6, 1999. See Example 19 on pages 58-59 of WO 93/09806.

The Examiner states, "It is further noted that the use of higher pH values (e.g. pH 7.4) than that utilized in the thionitrosylated hemoglobin example (e.g. pH 6.9 Example 19) is also suggested by the reference since thionitrosylated proteins are known to be stable under physiological conditions (e.g. TBS, pH 7.4, room temperature: see page 31) and further the reference discloses the use of pH 7.4 in the steps analogous to that of Example 19: see page 30, lines 20-27; page 33, lines 20-26)."

The reported stability of S-NO-t-PA at pH 7.4 does not reveal anything about the reaction conditions under which it can be made. WO 93/09806 does not disclose "the use of pH 7.4 in the

steps analogous to that of Example 19." On page 30, lines 20-27 (also on page 33, lines 20-26, for bovine serum albumin) of WO 93/09806 is reported a 3-step process:
Step 1: "t-PA was first dialyzed against *a large excess of 10 mM HCl* for 24 hours to remove excess L-arginine used to solubilize the protein." HCl is a strong acid. This step occurs at a pH much lower than 7.4.
Step 2: "t-PA was then exposed to NO_x generated from equimolar $NaNO_2$ in *0.5 N HCl (acidified $NaNO_2$)* or in control experiments, *to 0.5 N HCl alone*, for 30 minutes at 37°C." This second step is the step in which the nitrosylation occurs. HCl is used in both experimental and control conditions for the reaction. HCl is a strong acid. This step occurs at a pH much lower than 7.4 – probably about pH 2.
Step 3: "Solutions were titrated to pH 7.4 with equal volumes of 1.0 N NaOH and Tris Buffered Saline (TBS), pH 7.4, 0.05 M L-arginine." After the reaction has occurred under acidic conditions, the pH is raised to 7.4 using 1.0 N NaOH, a strong base. No reaction occurs at pH 7.4; no "NO_x" is generated from $NaNO_2$ at pH 7.4.
It can be seen from an analysis of these steps, that pH 7.4 is not used for any nitrosylation reaction. A pH higher than 6.9 is not suggested for any reaction described in WO 93/09806.

Rejection of Claims 17-19 and 29 Under 35 U.S.C. § 103(a) (Item 8 of Office Action)

Claims 17-19 and 29 have been rejected under 35 U.S.C. § 103(a), as they are said to be unpatentable over Feola *et al.*, U.S. Patent No. 5,439,882 and Stamler et al., (WO 93/09806), and, alternatively, further in view of Moore *et al.* (*J. Biol. Chem. 251(9)*:2788-2794, 1976 or Sharma *et al.* (*J. Biol. Chem. 253(18)*:6467-6472, 1978).

Feola *et al.* (US 5,439,882) describe a cross-linked mammalian hemoglobin, a method of making the same, and a method of using the same as a blood substitute. Reduced glutathione, a thiol that occurs naturally in red blood cells, is used in the method of synthesis to stop the cross-linking of hemoglobin when using o-adenosine as a cross-linking agent; in this case glutathione reacts through its amine group to become cross-linked to a second glutathione molecule or to become cross-linked to hemoglobin. *Excess glutathione is dialyzed out, so that the cross-linked hemoglobin composition contains no free low molecular weight thiol.* See column 13, lines 2-6 and lines 27-30, and column 18, lines 62-64. Glutathione, in any case, is not a low molecular weight *S-nitrosothiol*, as called for in the blood substitute of Claim 18. Feola *et al.* do not

suggest any advantage for including any S-nitrosothiol in a blood substitute, nor do the other cited references.

Claims 17-19 and 29 are drawn to methods requiring nitrosated (in the case of Claim 29, either nitrosated or nitrated) hemoglobin. Claim 18 also requires low molecular weight S-nitrosothiol in addition to nitrosated hemoglobin. The hemoglobin composition of Feola contains no nitrosated or nitrated hemoglobin, and no low molecular weight S-nitrosothiol at all. Feola *et al.* do not teach or suggest any form of nitrosated hemoglobin or suggest any advantage for it, or for the addition of a low molecular weight S-nitrosothiol. Nor do Feola *et al.* teach or suggest any form of a nitrosyl-heme-containing donor of NO, such as nitrosyl-hemoglobin.

The teachings of Stamler *et al.* (WO 93/09806) have been described above.

Sharma *et al.* (*J. Biol Chem. 253*:6467-6472, 1978) describe experiments to measure the rate of dissociation of NO from nitrosylhemoglobin. The rate constant is on the order of 10^{-4} or 10^{-5}, indicating that nitrosylhemoglobin is very stable, hence, cannot donate NO or have any physiological effect of an NO donor. The Moore *et al.* (*J. Biol. Chem. 251(9)*:2788-2794, 1976) reference describes experiments on nitrosylhemoglobin and nitrosylmyoglobin (both produced from the respective deoxy molecules), in which dissociation of NO from these molecules is followed spectrophotometrically in the absence of oxygen. Moore *et al.* find dissociation rate constants similar to those found by Sharma *et al.* The Moore *et al.* and Sharma *et al.* papers do not report or suggest any physiological effect of nitrosylhemoglobin or any other nitrosyl-heme containing NO donor. The low dissociation constant measured for nitrosylhemoglobin -- 1,000 times lower than that of CO and 200,000 times lower than that of oxygen, by comparison (see, for example, Greenburg, A.G. and H.W. Kim, *Art. Cells, Blood Subs., and Immob. Biotech. 23*:271-276, 1995, especially fifth paragraph on page 272; reference AX) – would lead one of ordinary skill in the art to conclude that nitrosylhemoglobin cannot be a donor of NO and that nitrosylhemoglobin could have no physiological effect because of the extremely low rate of release of NO from hemoglobin.

Blood substitutes are known in the prior art, but none containing a nitrosated or nitrated derivative of hemoglobin. Their administration to a human or other mammal to restore blood volume and restore capacity for carrying oxygen is known. An effect of hemoglobin-based blood substitutes has been vasoconstriction. See Feola *et al.*, last sentence of column 4. It is therefore desirable to limit any vasoconstrictive effect found in a hemoglobin-based blood substitute.

The Examiner states:

> The Stamler reference specifically discloses the use of nitrosylated proteins and low molecular weight nitrosating agents (e.g., see pages 1-2; page 24, lines 10-16) preparations thereof for the treatment of disorders by increasing oxygen capacity and transport; modulating CO and NO to tissues; scavenging radicals and vasodilation such as treating lung diseases (e.g. ARDS) and hypoxic disorders (e.g., see pages 19-25 and claims).

The Stamler reference discusses a method of producing SNO-hemoglobin which was unsuccessful, as explained previously. The reference shows no physiological effect of SNO-hemoglobin, as no SNO-hemoglobin was produced. The Stamler reference discussed certain effects of other SNO-proteins that were produced by a method using acidified nitrite. There was nothing about these reported effects of the SNO-proteins that could have suggested the effects observed with SNO-hemoglobin when it was ultimately made and tested. Unlike the SNO-proteins described in the Stamler reference, which act as vasodilators, SNO-hemoglobin is not in every case a vasodilator, and can be a vasoconstrictor in some cases. See, for example, page 17, lines 17-29, page 19, lines 12-25, page 57, line 3 to page 58, line 2 and Figure 4A.

One of ordinary skill in the art might have wanted to use SNO-oxyhemoglobin, if it could be made, as a component of a blood substitute for its expected oxygen carrying capacity and its expected vasodilatory effects, based on the effects of other SNO-proteins. However, one of ordinary skill in the art could not have predicted from the teachings of Stamler or from any combination of the cited references that SNO-hemoglobin could have the opposite -- that is, a vasoconstrictive -- effect, and therefore would not be desirable in methods of therapy in which vasoconstriction is undesirable (e.g., cardiovascular disorders or respiratory disorders as mentioned in Stamler *et al.*).

The Examiner states, "Further, nitrosylated hemoglobin preparations, e.g., nitrosylhemoglobin compositions, are conventionally known in the art. E.g., see the Moore and Sharma references." It is true that nitrosylhemoglobin was known. However, it was known to have a very high affinity for NO, and therefore would not be suspected of ever being a donor of NO itself, and would not be suspected of being converted to SNO-hemoglobin, which is a donor of NO. Nothing in the prior art suggests this conversion process.

Combining the teachings of Stamler *et al.* and Feola *et al.*, one of ordinary skill in the art might seek to use SNO-hemoglobin in a blood substitute, to deliver oxygen and NO, but would not know how to make it from Example 19 of Stamler *et al.*, and would have no reason to

combine it with a low molecular weight S-nitrosothiol, as in Claim 18. If one of ordinary skill in the art could obtain a sample of SNO-hemoglobin, he would be surprised that in the oxyhemoglobin form, SNO-hemoglobin would cause vasoconstriction, unlike other SNO-proteins. One of ordinary skill in the art would have no reason based on anything found in the cited references, including Moore *et al.* and Sharma *et al.*, to think of including nitrosylhemoglobin in a blood substitute, as nitrosylhemoglobin was not known to have any physiological effect. It was unknown until the subject application that nitrosylhemoglobin could be converted in the lung to SNO-hemoglobin, a donor of NO. See, for instance, Example 15, page 72, line 21 to page 73, line 8, Example 18, page 75, lines 5-28, and page 28, line 34 to page 29, line 5.

Rejection of Claims 24-26 Under 35 U.S.C. § 103(a) (Item 9 of Office Action)

Claims 24-26 have been rejected under 35 U.S.C. § 103(a), as they are said to be unpatentable over Feola *et al.* and Stamler, and alternatively, over Feola *et al.* and Stamler *et al.*, further in view of Moore or Sharma and Wade and Castro.

Claims 24-26 have been canceled.

CONCLUSION

The Examiner is respectfully requested to consider the above amendments and remarks, and to withdraw the remaining rejections. If after consideration of this Amendment, the Examiner has not found all the claims to be allowable, he is requested to call the undersigned attorney so that an interview can be arranged.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By Carol A. Egner

Carol A. Egner
Registration No. 38,866
Telephone (781) 861-6240
Facsimile (781) 861-9540

Lexington, Massachusetts 02421-4799
Dated: October 31, 2000

969.

Oxidation of Nitrogen Oxides by Bound Dioxygen in Hemoproteins

Michael P. Doyle and James W. Hoekstra

Department of Chemistry, Hope College, Michigan

ABSTRACT

Nitric oxide is unique among the higher oxides of nitrogen in its reactivity and efficiency for the oxidation of oxygen-bound hemoproteins. Dinitrogen trioxide serves as a nitric oxide donor, but dinitrogen tetroxide does not exhibit similar reactivity. Details are provided of the stoichiometric transformation through which nitric oxide is converted to nitrate with accompanying oxidation of myoglobin or hemoglobin to the corresponding iron(III) hemoprotein, including an estimate of the rate constant for nitric oxide oxidation of oxygen-associated myoglobin and the effect of unassociated oxygen on the stoichiometry and rates for nitric oxide oxidation. Evidence is presented to establish the mechanism of oxidation in the direct combination of nitric oxide with iron(II)-bound dioxygen.

INTRODUCTION

Although the gaseous nitrogen oxides NO, N_2O_3, and N_2O_4 are well-known atmospheric pollutants and potential health hazards [1–5], the chemistry of their action on molecules of biological significance has received scant attention. In particular, the interactions of these nitrogen oxides with the oxygen-carrying hemoproteins, hemoglobin and myoglobin, have not been investigated despite evidence from in vivo studies that nitric oxide oxidizes hemoglobin to methemoglobin [4, 6] and also leads to the formation of nitrosylhemoglobin [7, 8]; nitrogen dioxide toxicity is recognized to result, at least in part, from the production of methemoglobin and bilirubin which causes decreased oxygen transport capability [9]. The reactivity of nitric oxide and dinitrogen trioxide with molecular oxygen [10] and the lack of specific information on the reactions of nitrogen oxides with mononuclear dioxygen complexes [11] has limited interpretation of results obtained with oxygenated hemoproteins. The present investigation of the reactions of nitrogen oxides with hemoglobin and myoglobin was initiated as part of a general program to provide a molecular understanding of the reactions of nitrogen oxides with metalloproteins. Because hemoglobin and myoglobin exhibit optimum

Address reprint requests to Dr. Michael P. Doyle, Department of Chemistry, Hope College, Holland, MI 49423.

Journal of Inorganic Biochemistry 14, 351-358 (1981)

52 Vanderbilt Ave., New York, New York 10017

0162-0134/81/040351-0852.50

oxygen-binding capability, they can also be regarded as the systems most suitable for model studies of similar reactions with mononuclear dioxygen complexes.

EXPERIMENTAL

Human hemoglobin A (type IV) and equine heart myoglobin (type III) (Sigma Chemical Co.) were reduced with excess sodium dithionite and further purified by passing the resulting aqueous solutions through a G-25 Sephadex column using 0.05 M phosphate buffer. Solutions of oxymyoglobin were degassed under reduced pressure (less than 0.5 Torr) without evidence of deoxygenation of the hemoprotein. Similar treatment of oxygenated solutions of hemoglobin was continued until a mixture of deoxy- and oxyhemoglobin was obtained. Individual solution concentrations of these hemoproteins were determined spectroscopically. Nitric oxide (99%) and dinitrogen tetroxide (99.5%) (Union Carbide Corp., Linde Division) were used without further purification. Commercial dinitrogen trioxide (99.5%) (Union Carbide) was purified by the method of Challis and Kyrtopoulos [12].

Reactions of the gaseous nitrogen oxides with oxygenated myoglobin and hemoglobin were performed in phosphate-buffered solutions (0.05 M, pH 7) of the hemoprotein ($5\text{-}17 \times 10^{-6}$ M). Degassed hemoprotein solutions were maintained at atmospheric pressure under nitrogen in sealed air-tight flasks. The atmosphere above hemoprotein solutions saturated with molecular oxygen was flushed with nitrogen prior to addition of the nitrogen oxide. Predetermined volumes of the nitrogen oxides were injected into the reaction solution from a gas-tight syringe with rapid mixing of the resultant solution. Alternatively, solutions of nitric oxide in deoxygenated water (2.6×10^{-3} M) were injected into the hemoprotein reaction solution without noticeable variation in the results obtained.

The concentrations of nitrate produced in these reactions were determined through the use of the Orion 93 series nitrate-specific ion electrodes. A calibration curve was determined for each set of experiments from standard nitrate solutions in 0.5 M phosphate buffer. Interferences from nitrite ion and from the hemoproteins were determined and found to be negligible. Analysis of nitrite through the use of the Orion Model 95-46 nitrogen oxide electrode was not sufficiently sensitive to conclusively determine low levels of nitrite under these conditions (less than 10% of the reactant hemoprotein or nitrogen oxide concentration); however, the absence of nitrite ion levels above 0.10 times the hemoprotein concentration in stoichiometric studies was established. Additional confirmation of the identity of nitrate and determination of its yield in stoichiometric reactions of nitrogen oxides with hemoproteins was obtained by HPLC analyses [13]. Concentrations of hemoprotein constituents were determined spectrophotometrically.

For accurate concentration measurements in kinetic determinations, reactions with N_2O_3 and N_2O_4 were performed by injecting a standardized concentrated acetonitrile solution of these nitrogen oxides into the hemoprotein reaction solution. The volume of acetonitrile added to the phosphate-buffered hemoprotein solutions was less than 2% of the total volume of the reaction solution. Rates of oxidation were determined at 25.0°C by monitoring the decrease in absorbance at 576 nm with time. A minimum of four kinetic runs was used to determine the rate of oxidation. Initial hemoprotein concentrations were within the range $0.5\text{-}1.1 \times 10^{-4}$ M; initial nitrogen oxide concentrations were $0.4\text{-}2.0 \times 10^{-4}$ M. Spectral confirmation of quantitative oxidation was obtained following each kinetic determinati n.

RESULTS AND DISCUSSION

Nitric Oxide

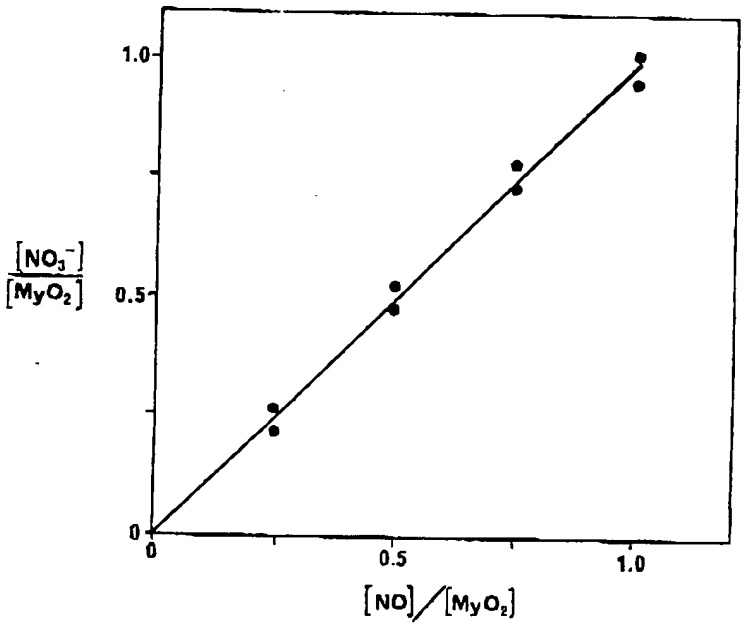
The addition of nitric oxide to degassed solutions of oxymyoglobin (MyO_2) in 0.05 M phoaphate buffer (pH = 7) resulted in the immediate formation of oxidized hemoprotein, metmyoglobin (My^+), and in the production of nitrate. Results from the addition of defined molar amounts of nitric oxide to oxymyoglobin solutions, which are presented in Figure 1, describe the net stoichiometry of this reaction as

$$MyO_2 + NO = My^+ + NO_3^-. \quad (1)$$

Nitrate concentrations were directly measured immediately following the introduction of nitric oxide to the reaction solutions, and $[My^+]/[MyO_2]$ molar ratios were determined from identical reaction solutions in separate experiments. Metmyoglobin/oxymyoglobin molar ratios in these stoichiometric studies corresponded to the separately determined $[NO_3^-]/[MyO_2]$ ratios. Similar results were observed for partially deoxygenated solutions containing oxyhemoglobin and hemoglobin; in these experiments, the proportion of methemoglobin (Hb^+) to nitrosylhemoglobin (HbNO) corresponded closely to the initial ratio of $[HbO_2]$ to [Hb] (±5%).

The reactions of nitric oxide with either MyO_2 or HbO_2 are complete, within the limits of our measurement capabilities, in less than 2 sec at 25°C. Indeed, the virtual absence of MyNO (<1–4%) and the close correspondence of $[Hb^+]/[HbNO]$ to the initial $[HbO_2]/[Hb]$ in stoichiometric reactions of nitric oxide with degassed solutions of MyO_2 and HbO_2/Hb attests to the rapid rate of the nitric oxide oxidation process. These results suggest that the rate of oxidation of the oxygen-carrying hemoprotein by nitric oxide is, in fact, competitive with the rates for association and dissocia-

FIGURE 1. Molar ratios of nitrate to reactant MyO_2 formed from reactions of nitric oxide with MyO_2 in degassed, phosphate-buffered solutions. Data points are limiting nitrate determinations.



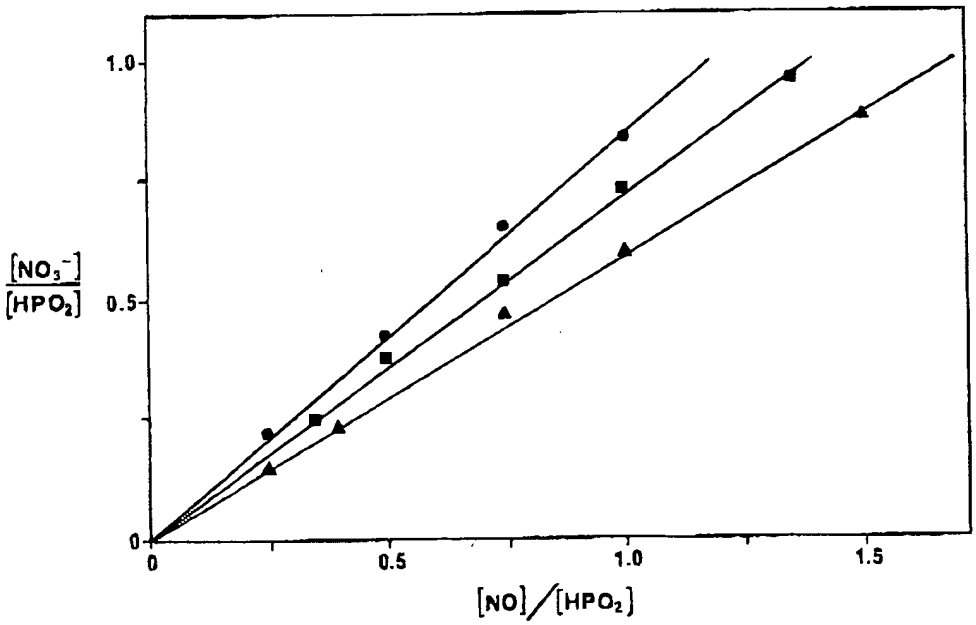
tion of molecular oxygen [14, 15] and that, to a first approximation, measurement of the molar ratio of oxidized hemoprotein (HP^+) to nitrosylhemoprotein (HPNO) may afford a reliable indirect measure of the equilibrium concentrations of the oxygen-associated hemoprotein (HPO_2) and the deoxygenated hemoprotein (HP):

$$\begin{array}{ccc} HPO_2 & \rightleftharpoons & HP + O_2 \\ \Big\downarrow{\scriptstyle NO} & & \Big\downarrow{\scriptstyle NO} \\ HP^+ & & HPNO \end{array}$$

Reactions of nitric oxide with HbO_2 and with MyO_2 in the presence of molecular oxygen provided the results described in Figure 2. The oxygen concentration in these reaction solutions were maintained at saturation (1.2 mM) in the phosphate-buffered solutions, and variable concentrations of the hemoproteins were employed. Reactions were performed with nitric oxide concentrations adjusted to the reactant hemoprotein concentration, and molar ratios were based on heme-iron(II) equivalents. As described in Figure 2, oxidation of either HbO_2 or MyO_2 is competitive with the direct reaction of nitric oxide with molecular oxygen, even though association of nitric oxide with molecular oxygen [16, 17] could have been expected either to change the nature of the oxidative process or to markedly slow the rate of hemoprotein oxidation. These results allow us to set the lower limit for the rate of HbO_2 oxidation by nitric oxide as approximately 2.6×10^1 faster than the rate of oxidation of nitric oxide by molecular oxygen from the following equation:

$$\frac{[NO_3^-]^{HbO_2+NO}}{[NO_3^-]^{NO+O_2}} = \left(\frac{k^{HbO_2+NO}}{k^{NO+O_2}}\right)\frac{[HbO_2]}{[O_2]}.$$

FIGURE 2. Molar ratios of nitrate to reactant HPO_2 from reactions of nitric oxide with HbO_2 and MyO_2 in oxygen-saturated, phosphate-buffered solutions: •, 1.0×10^{-4} M HbO_2; ▪, 5.0×10^{-5} M HbO_2; ▲, 6.0×10^{-5} M MyO_2. Average values for nitrate determinations are presented.



The assumptions made in formulating this equation, i.e., a first-order dependence on oxygen concentration for the reaction of nitric oxide with molecular oxygen and of the direct relationship between product ratios and rate ratios, allow only an approximation of the actual competitive events. However, these results do specify that, even in oxygen-saturated solutions, the action of nitric oxide on oxygen-associated hemoproteins is direct and should not be considered to result from a prior conversion of nitric oxide to an alternate oxidant such as nitrite [6]. Similarly, since the actual effect of nitric oxide on HbO_2 is oxidative conversion to Hb^+, rather than oxygen replacement by nitric oxide resulting in the formation of HbNO, recent findings that only relatively constant low levels of HbNO are observed upon exposure of human subjects to variable low levels of nitric oxide [8] are understandable.

The abnormally rapid oxidation of HbO_2 and MyO_2 by nitric oxide is consistent with the recent proposal that these oxygenated hemoproteins serve as superoxide donors [18, 19]. Although we are not able to identify the specific sequence of molecular events in these reactions, conversion of nitric oxide by the oxygenated hemoprotein to peroxynitrite, followed by isomerization of peroxynitrite to nitrate [20, 21]

$$HPO_2 + NO \rightarrow HP^+ + {}^-OONO \tag{2}$$

$$ONOO^- \rightarrow NO_3^- \tag{3}$$

would appear to be the likely mechanism for these oxidations. Since nitric oxide is known to have access to the heme pocket of myoglobin and hemoglobin [15], this intimate electron transfer process represents a reasonable alternative to the outer-sphere electron transfer mechanism proposed by Wallace and Caughey [19] for phenol oxidations of oxyhemoglobin.

Dinitrogen Trioxide

Like nitric oxide, dinitrogen trioxide rapidly oxidizes HbO_2 and MyO_2 with a net stoichiometry consistent with the following equation:

$$HPO_2 + N_2O_3 = HP^+ + NO_3^- + NO_2. \tag{4}$$

Reactions of degassed solutions of oxymyoglobin with N_2O_3 ($[MyO_2] = [N_2O_3] = 9.5 \times 10^{-5}$ M) under a nitrogen atmosphere exhibit rapid conversion to metmyoglobin with a half-life of approximately 7 sec at 25.0°C ($k_2 = 27 \pm 7 \times 10^2$ M^{-1} sec^{-1}). Second-order kinetics, first order in $[MyO_2]$ and first order in $[N_2O_3]$, are observed for this transformation through five half-life periods. In oxygen-saturated solutions stoichiometric oxidation is also observed, but the half-life for conversion of MyO_2 to My^+ is approximately 50 sec ($k_2 = 4.1 \pm 0.3 \times 10^2$ M^{-1} sec^{-1}). The slower rate of oxidation in oxygen-saturated solutions is consistent with nitrogen trioxide formation from oxygen association with nitric oxide. The nitrogen trioxide radical has been frequently postulated in reaction mechanisms for reactions of NO with O_2 [10, 22] and has recently been detected in gas-phase reactions of NO with O_2 at low pressures [23]. Similar reactions of HbO_2 with N_2O_3 in oxygen-saturated, phosphate-buffered (pH 7) solutions exhibited second-order kinetics, first order in HbO_2 and first order in N_2O_3, with a rate constant of $4.7 \pm 0.3 \times 10^2$ M^{-1} sec^{-1} at 25.0°C.

The composite results suggest a mechanism for oxidation by N_2O_3 that involves initial nitric oxide dissociation from N_2O_3,

$$N_2O_3 \rightleftharpoons NO + NO_2 \quad (5)$$

$$NO + O_2 \rightleftharpoons ONOO \quad (6)$$

$$NO + HPO_2 \rightarrow HP^+ + NO_3^- \quad (7)$$

That N_2O_3 is not the actual oxidant is implied by the observation of effective inhibition of oxidation by molecular oxygen. Dissolved molecular oxygen is proposed to slow the rate of hemoprotein oxidation through formation of the peroxynitrite radical, but it is unlikely that this unstable species is a contributing oxidant for the conversion of HPO_2 to HP^+.

In aqueous solution, N_2O_3 exists predominantly in the associated state ($K_{N_2O_3}$ = 7.3 X 10^{-5} M) [24]. Recombination of NO with NO_2 is characterized by a rate constant of 1.1 X 10^9 M^{-1} sec^{-1} at 20°C [24]. Thus the concentration of NO in the oxidative reactions performed with N_2O_3 is approximately four orders of magnitude less than in the previously described reactions of NO with oxygen-associated hemoproteins. As a result of its relatively low concentration, nitric oxide dissociated from N_2O_3 can be expected to associate competitively with O_2 without undergoing subsequent conversion to N_2O_4,

$$ONOO + NO \rightarrow N_2O_4, \quad (8)$$

as is observed in reactions performed with nitric oxide directly. Consequently, N_2O_3 undergoes stoichiometric oxidation of oxygen-associated hemoproteins, even in oxygen-saturated solutions; whereas nitric oxide introduced at the higher concentration levels is competitively oxidized to N_2O_4.

If, as our results imply, NO is the actual oxidant of HPO_2 in reactions with N_2O_3, the observed rate constant can be approximated as a composite of the rate constant for reaction (7), $k_{(7)}$, and the equilibrium constant for dissociation of NO from N_2O_3 (reaction 5). Calculated in this manner, $k_{(7)}$ is equal to 37 X 10^6 M^{-1} sec^{-1} for the oxidation of myoglobin at 25°C. The value of this rate constant corresponds closely to kinetic values recently obtained for the association of oxygen and nitric oxide with myoglobin and hemoglobin [15, 25, 26] and confirms our earlier suggestion that the rate of oxidation of MyO_2 and HbO_2 by NO is competitive with the rates of association and dissociation of molecular oxygen [14, 15].

Dinitrogen Tetroxide

Like N_2O_3, N_2O_4 exists predominantly in its dimeric state in aqueous solutions ($K_{N_2O_4}$ = 1.53 X 10^{-5} M) [27]. Unlike N_2O_3, however, N_2O_4 reacts slowly with HbO_2 to form Hb^+. At a 1:1 ratio of N_2O_4 to HbO_2 in oxygen-saturated solutions, less than 10% oxidation occurs within 60 min at 25°C. At molar ratios of N_2O_4/HbO_2 equal to between 5 and 10, initial slow oxidation is observed for between 60 and 300 sec followed by rapid autooxidation, whose onset is dependent on the initial N_2O_4/HbO_2 molar ratio. The kinetic characteristics of this oxidation are remarkably similar to those reported for nitrite oxidations of oxygen-associated hemoglobin under

the same conditions [18, 28, 29]. This similarity suggests that nitrite, formed by hydrolysis of N_2O_4, rather than N_2O_4 or NO_2,

$$N_2O_4 + H_2O \rightleftharpoons NO_2^- + NO_3^- + 2H^+, \quad (9)$$

is the principal oxidant under these coniditions. Further delineation of the nature of this oxidative process awaits elucidation of the complex mechanism for nitrite oxidations of deoxy- and oxygen-associated hemoglobin, an effort that we are currently undertaking.

The fate of nitrogen dioxide in reactions of N_2O_3 with oxygen-bound hemoproteins can be estimated from these results. Dimerization of NO_2 to N_2O_4, a reaction that occurs with a specific rate defined by $k = 4.5 \times 10^8$ M^{-1} sec^{-1} at 20°C in water [27], is the expected result. The contribution by NO_2 or N_2O_4 to the overall N_2O_3 oxidative process (reaction 4) is negligible. Nitric oxide is the unique oxidant among the nitrogen oxides.

We gratefully acknowledge support of this work by the U.S. Public Health Service (Grant ES 01673). We are grateful to the National Science Foundation for their URP award to J. W. H. for the summer of 1979.

REFERENCES

1. Environmental Protection Agency, *Air Quality Criteria for Nitrogen Oxides*, AP-84, Washington, DC (1971).
2. T. L. Guidotti, *Environ. Res.* 15, 443 (1978).
3. C. Hugod, *Int. Arch. Occup. Environ. Health* 42, 159 (1979).
4. K. Kon, N. Maeda, and T. Shiga, *J. Toxic. Environ. Health* 2, 1109 (1977).
5. J. R. Rowlands and E. M. Gause, *Arch. Intern. Med.* 128, 94 (1971).
6. E. Azoulay, L. Lachia, M.-C. Blayo, and J.-J. Pocidalo, *Toxicol. Europ. Res.* 1, 7 (1978).
7. H. Oda and S. Kusumoto, *Arch. Environ. Health* 30, 453 (1975).
8. G. Freeman, R. L. Dyer, L. T. Juhos, G. A. St. John, and M. Anbar, *Arch. Environ. Health* 33, 19 (1978).
9. R. A. Ehrman, M. Treshow, and I. M. Lytle, *Am. Indust. Hygiene Assoc. J.* 751 (1972).
10. I. R. Beattie, in *Mellor's Comprehensive Treatise on Inorganic and Theoretical Chemistry*, Vol. 8, Suppl. II, Wiley, New York, 1967, Sec. XXII.
11. G. McLendon and A. E. Martell, *Coord. Chem. Revs.* 19, 1 (1976).
12. B. C. Challis and S. A. Kyrtopoulos, *J. C. S., Perkin Trans. I* 299 (1979).
13. R. G. Gerritse, *J. Chromatography* 171, 527 (1979).
14. C. A. Sawicki and Q. H. Gibson, *J. Biol. Chem.* 252, 7538 (1977).
15. K. Moffat, J. F. Deatherage, and D. W. Seybert, *Science* 296, 1035 (1979).
16. J. N. Pitts and A. C. Lloyd, in *Nitrogenous Compounds in the Environment* (Report of the U.S. Environmental Protection Agency Hazardous Materials Advisory Committee), U.S. Govt. Printing Office, Washington, DC, 1973, p. 43.
17. A. Treinin and E. Hayon, *J. Am. Chem. Soc.* 92, 5821 (1970).
18. W. J. Wallace, J. C. Maxwell, and W. S. Caughey, *Biochem. Biophys. Res. Commun.* 57, 1104 (1974).
19. W. J. Wallace and W. S. Caughey, *Biochem. Biophys. Res. Commun.* 62, 561 (1975).
20. M. Anbar and H. Taube, *J. Am. Chem. Soc.* 76, 6243 (1954).
21. J. D. Ray, *J. Inorg.Nucl. Chem.* 24, 1159 (1962).
22. J. D. Ray and R. A. Ogg, Jr., *J. Chem. Phys.* 26, 984 (1957).
23. W. A. Guillory and H. S. Johnson, *J. Chem. Phys.* 42, 2457 (1965).
24. M. Gratzel, S. Taniguchi, and A. Henglein, *Ber. Bunsenges. Phys. Chem.* 74, 488 (1970).

25. E. G. Moore and Q. H. Gibson, *J. Biol. Chem.* **251**, 2788 (1976).
26. R. Cassoly and Q. H. Gibson, *J. Mol. Biol.* **91**, 301 (1975).
27. M. Gratzel, A. Henglein, J. Lilie, and G. Beck, *Ber. Bunsenges. Phys. Chem.* **73**, 646 (1969).
28. J. H. Austin and D. L. Drabkin, *J. Biol. Chem.* **112**, 67 (1935).
29. F. L. Rodkey, *Clin. Chem.* **22**, 1986 (1976).

Received June 23, 1980; accepted November 20, 1980